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# Intercalibrations of freshwater phytoplankton analyses

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Phytoplankton is one of the biological quality elements of the Water Framework Directive of European Union for ecological classification of surface waters included. The reliability and comparability of phytoplankton data is, therefore, essential in assessment of the classification. The recently published standard for phytoplankton analysis based on the Utermöhl technique supports stringent demands for comparability of data. We tested the comparability of phytoplankton analyses (Utermöhl method) between professional and non-professional analysts. The coefficient of variation was highly variable, 10%–42% for professionals and 8%–57% for non-professionals. Elimination of the error caused by subsampling and the increase in the minimum number of enumerated units decreased the CV especially within professionals. CV was smaller (4%–19%) when samples were counted by one person. In order to minimize the variability caused by differences between subsamples, new methods for comparison testing both for technical enumeration and for species identification should be developed.

## Introduction

Phytoplankton communities are sensitive to changes in their environment and, therefore, phytoplankton total biomass and many phytoplankton species are used as indicators of water quality (Järnefelt 1952, Heinonen 1980, Olrik 1994, Reynolds 1997, Heinonen *et al*. 2000, Reynolds *et al*. 2002, Brettum and Andersen 2005). Phytoplankton communities give more information on changes in water quality than mere nutrient concentrations or chlorophyll *a* concentrations. Standards for chemical laboratory analyses (e.g. National Board of Waters in Finland 1981 and 1984, SNV Rapport 3075), which are relatively simple to follow, have been used since the 1980s. In contrast, quantitative analyses of natural phytoplankton multispecies communities are more complicated to perform. Furthermore, only recently a commonly accepted standard for phytoplankton enumeration was published (EN 15402 2006).

Currently phytoplankton counting methods vary from country to country. Several alternative counting chambers, e.g. Sedgwick-Rafter, Lund and Palmer-Maloney, are used for phytoplankton analyses if inverted microscopes are not available (Welch 1948, Palmer and Maloney 1954, Lund *et al*. 1959, Guillard 1978). The Sedgwick-Rafter chamber is best suited for counting large algae

at low concentrations, and the Palmer-Maloney chamber is of use for nanoplankton counting (Guillard 1978, EN 15402 2006). The Utermöhl technique (Utermöhl 1958), however, is better suited in quantitative analyses of multispecies phytoplankton communities, especially in nutrient-poor waters. This method requires an inverted microscope equipped with high quality optics in order to obtain reliable results (Olrik *et al*. 1998, Lepistö 1999). Although a uniform methodology for phytoplankton counting based on Utermöhl method was suggested by Olrik *et al*. (1998) for freshwater, the recommended method is not fully followed or evaluated. The new European standard for phytoplankton counting and statistical analyses based on Utermöhl technique has been published (EN 15402 2006) and should replace conflicting national standards in 29 European countries at the latest by February 2007.

The implementation of the Water Framework Directive (European Union 2000) requires frequent phytoplankton monitoring and comparable phytoplankton results, e.g. the use of equal counting methods and harmonization of species identification, at least among members of the same Geographical Intercalibration Group (GIG). Ecological classification of surface waters included in the Water Framework Directive is based not only on quantities of phytoplankton (biomass) but also on taxonomic composition and size structures of phytoplankton communities. At the present, phytoplankton analysis is the only method to produce these taxonomic quality elements, including e.g. indicator species. Harmonization of enumeration procedures and species identification should, therefore, be included in the quality control of phytoplankton analyses.

During the last decades, phytoplankton research quality controls in brackish water have been introduced among Baltic countries (HELCOM 1997). For freshwaters, however, quality controls of phytoplankton analyses are still rare, despite the fact that phytoplankton communities have been studied in Nordic countries since the beginning of the 20th century (in Finland e.g. Levander 1900, Blomqvist *et al*. 1915, Järnefelt 1937, and in Sweden e.g. Cleve-Euler and Huss 1912). Furthermore, the nation-wide routine phytoplankton monitoring programmes have been operated by Finnish and Swedish authorities since the 1960s (e.g. Heinonen 1980, Willén 2001, Lepistö 2004). Since the 1980s Finnish phytoplankton analysts as well as the Nordic phytoplankton and periphyton group (NPPG) have harmonized species identification. Recently, intercalibrations of phytoplankton enumerations have also been introduced — e.g. in 1989 the Danish National Environmental Monitoring Programme for lakes was launched including routine intercalibrations among active Danish phytoplankton analysts (Jensen and Søndergaard 1994, Lauridsen *et al*. 2005).

Performed intercalibrations have shown that there are differences in phytoplankton results between laboratories as well as between individual phytoplankton counts carried out by different persons (Barinova *et al*. 1980, Rott 1981, Huttunen 1985, Niemi *et al*. 1985, Jensen and Søndergaard 1994, HELCOM 1997). This must be taken into account when comparing phytoplankton results. Though in Nordic countries the Utermöhl technique is widely used (Olrik *et al*. 1998), every individual analyst modifies this technique according to the properties of the microscope used.

There is thus an urgent need to reconsider phytoplankton analysis methodology, to train analysts, and to perform intercalibrations among working analysts to increase the accuracy of phytoplankton data. Furthermore, for ecological classification there is a need to determine the acceptable level of variation caused by differences in subsamples. In this paper we provide results from two intercalibrations among Finnish universities and research laboratories, and one joint Finnish–Swedish–Norwegian intercalibration. The aim of these intercalibrations was to evaluate the identification of species, and, more importantly, variability of quantitative results. Here we discuss the variability of counting results between analysts, and methodological problems related to intercalibrations.

## Methods

Three intercalibration sessions were performed in 2002, 2003 and 2005. Participants were divided into two groups, and the results of phytoplankton analyses performed by professional analysts

were, on daily basis, compared with those of analysts occasionally counting phytoplankton samples or students in training to be professional phytoplankton analysts. A nonparametric Mann-Whitney *U*-test was used to compare differences between professional and non-professional phytoplankton analysts.

### First intercalibration session 2002

The first intercalibration session was also a training session in the methodology, and was thus deliberately kept simple. The phytoplankton sample for this session represented a 2-meter-deep water column of a lake in SW Finland. Samples of 200 ml were preserved with acid Lugol's solution and combined into one sample of two litres. This combined sample was mixed and divided into subsamples of 100 ml for each participant. The two taxa to be counted were the cryptophyte *Rhodomonas lacustris* and the colony forming diatom *Fragilaria crotonensis*. In addition, only the volume to be sedimented (25 ml) was provided. Otherwise, every participant used their own modification of the Utermöhl counting method (Utermöhl 1958, Olrik *et al*. 1998). Participants were instructed to give the cell numbers as units per litre. Altogether 13 analysts participated in the first intercalibration.

### Second intercalibration session 2003

For the second intercalibration, the preparation of the sample was equal to the first session, but instructions were given in more detail. The settled volume was also 25 ml, and four taxa were to be counted: the diatoms *Aulacoseira subarctica* var. *subborealis* and *Asterionella formosa*; the chlorophyte *Desmodesmus bicellularis*; and the cyanobacterium *Radiocystis geminata*. *A. subarctica* var. *subborealis* were counted as single cells and *D. bicellularis* as cell pairs, using a total magnification of 600–1000×. *Asterionella* was also counted as single cells but *Radiocystis* as subcolonies containing approximately 150 cells using a total magnification of 200–250×. At least 50 units (i.e. cells, cell pairs or subcolonies) of each taxon were counted. Special attention was paid to the counting of colony forming taxa, therefore no attempt was made to split up the *Radiocystis* into single cells. Results were given as units per one litre. A total of 12 analysts took part in the second intercalibration.

In order to study the reproducibility of phytoplankton counts, Toini Tikkanen sedimented three subsamples and counted each of them using two different counting methods. The cryptophyte *Rhodomonas lacustris* was counted in crossing diagonals and in 20 fields with a total magnification of 500×. The diatom *Fragilaria crotonensis* was counted using the same methods but with a magnification of 200×. A nonparametric Mann-Whitney *U*-test was performed to test the differences between subsamples and between the two different counting methods (SPSS 11.5.1 for Windows, SPSS Inc. 1989–2002).

### Third intercalibration session 2005

The joint Finnish–Swedish–Norwegian intercalibration in 2005 was conducted during a Nordic phytoplankton workshop. The setup was simpler than that of a natural sample because only three laboratory strains were used, a unicellular green alga and two filamentous cyanobacteria. Two separate samples were settled, one containing the chlorophyte *Selenastrum* sp. and the other containing the two strains of filamentous cyanobacteria *Planktothrix* sp. and *Anabaena* sp. Participants were instructed to count 10 fields from one diagonal; *Selenastrum* sp. was counted as single cells and the two cyanobacteria as filaments. Analysts were also advised to assess the number of cells of *Anabaena* filaments and the number of 25 $\mu$m long subfilaments of *Planktothrix*. Magnifications were 650× for *Selenastrum* sp. and 400× for the cyanobacterial trichomes. All 22 participants counted the sample containing *Selenastrum* sp., but because of limited time only 14 participants counted both samples.

## Results

### Intercalibrations

During the first intercalibration, the range of

cell numbers counted was large: 23–253 cells of *Rhodomonas lacustris* and 450–2255 cells of *Fragilaria crotonensis*. The average cell numbers per litre are shown in Table 1. Among all participants, the coefficient of variation (CV) for both taxa was 21%. Among professionals ($N = 8$) who count phytoplankton samples daily, the CV was 19% for both *Rhodomonas* and *Fragilaria*. Among other analysts the corresponding value was 24%. The differences in phytoplankton counts between professionals and non-professionals were not statistically significant (Mann-Whitney *U*-test).

During the second intercalibration, the range of the cell number counted of *Aulacoseira* was 129–623, and of *Desmodesmus* 74–443. The range of colonies of *Radiocystis* was 55–166 and of *Asterionella* 80–1109. Table 2 gives the results converted into number of cells or colonies $l^{-1}$. When the cell and colony counts were converted into numbers of units $l^{-1}$, the coefficients of variation were 15% for *Aulacoseira*, 34% for *Desmodesmus*, 29% for *Radiocystis* and 35% for *Asterionella* (Table 2). The variation between participants was smaller than in the first intercalibration attempt: coefficients of variation among the professionals ($N$ = 7–8) were 14% for *Aulacoseira*, 19% for *Desmodesmus*, 33% for *Radiocystis*, and 42% for *Asterionella;* and 20, 57%, 23% and 20% for non-professionals, respectively. The phytoplankton counts between professionals and non-professionals were not statistically significant (Mann-Whitney *U*-test).

During the third intercalibration, the range of the counts of *Selenastrum* cells was 340–495 (CV = 9%, $N = 22$). For *Anabaena* the number of

**Table 1**. Results of the first intercalibration: individual unit counts and mean values (units $l^{-1}$), coefficients of variation (CV% = standard deviation as a percentage of the mean values) among professionals and among all participants. Taxa that were counted are *Rhodomonas lacustris* (Rhod lac) and *Fragilaria crotonensis* (Frag cro). Participant number 13 counted the *Rhodomonas lacustris* cells twice (= 13A and 13B).

| Counter no. | Rhod lac | Frag cro |
|---|---|---|
| 1 | 31200 | 163626 |
| 2 | 23664 | 140461 |
| 3 | 27988 | 139700 |
| 4 | 33600 | 132700 |
| 5 | 29600 | 186400 |
| 6 | 30400 | 68815 |
| 7 | 44020 | 219360 |
| 8 | 29268 | 177210 |
| 9 | 21726 | 140178 |
| 10 | 33300 | 250000 |
| 11 | 30666 | 180761 |
| 12 | 38500 | 158000 |
| 13 | 43314 | 157635 |
| Professionals | | |
| Mean | 31180 | 165637 |
| CV% | 19 | 19 |
| *N* | 8 | 8 |
| Non-professionals | | |
| Mean | 33501 | 177381 |
| CV% | 24 | 24 |
| *N* | 5 | 5 |
| All | | |
| Mean | 32073 | 170530 |
| CV% | 21 | 21 |
| *N* | 13 | 13 |

**Table 2**. Results of the second intercalibration: individual unit counts and mean values (units $l^{-1}$), coefficients of variation (CV% = standard deviation as a percentage of the mean values) among professionals and among all participants. Taxa that were counted are *Aulacoseira subarctica* var. *subborealis* (Au su;su), *Desmodesmus bicellularis* (Desm bic), *Radiocystis geminata* (Radi gem) and *Asterionella formosa* (Aste for).

| Counter no. | Au su;su | Desm bic | Radi gem | Aste for |
|---|---|---|---|---|
| 1 | 3540000 | 6020000 | 99670 | 90110 |
| 2 | 3409000 | 5382000 | 51360 | 54640 |
| 3 | 3712000 | 5184000 | 79880 | 25990 |
| 4 | 3470000 | 4400000 | 48000 | 49500 |
| 5 | 3540000 | 4980000 | 43200 | 38100 |
| 6 | 4346100 | 3951000 | 55800 | 52200 |
| 7 | 3588145 | 6960138 | 79128 | 47100 |
| 8 | 2585024 | | 46107 | 27072 |
| 9 | 4072000 | 3357000 | 44600 | 73700 |
| 10 | 3700000 | 3500000 | 64125 | 46170 |
| 11 | 2788378 | 7068214 | 80384 | 54636 |
| 12 | 2710400 | 1760000 | 60995 | 59886 |
| Professionals | | | | |
| Mean | 3523784 | 5268163 | 62893 | 48089 |
| CV% | 14 | 19 | 33 | 42 |
| *N* | 8 | 7 | 8 | 8 |
| Non-professionals | | | | |
| Mean | 3317695 | 3921304 | 62526 | 58598 |
| CV% | 20 | 57 | 23 | 20 |
| *N* | 4 | 4 | 4 | 4 |
| All | | | | |
| Mean | 3455087 | 4778396 | 62771 | 51592 |
| CV% | 15 | 34 | 29 | 35 |
| *N* | 12 | 11 | 12 | 12 |

cells counted varied from 297 to 542 (CV = 18%, $N = 14$) and that of trichomes from 43 to 99 (CV = 25%, $N = 13$). The range in *Planktothrix* counting units of 25 $\mu$m was 81–175 (CV 25%, $N = 14$) and that of whole trichomes from 35 to 67 (CV = 17%, $N = 13$). Among professional phytoplankton analysts, the deviations of CV were 10% for *Selenastrum* cells, 18% for *Anabaena* cells and 23% for *Anabaena* trichomes, 20% for *Planktothrix* counting units and 13% for *Planktothrix* trichomes (Table 3). For other analysts the corresponding deviations of CV were 8%, 18%, 16%, 35% and 20%, respectively. Again, the differences in phytoplankton counts between professionals and non-professionals were not statistically significant (Mann-Whitney *U*-test).

## Repeatability of phytoplankton countings

In order to test the reproducibility of the phytoplankton countings, two different subsamples of the same material were sedimented and counted three times by the same person, using two different methods (20 fields versus crossing diago-

**Table 3**. Results of the third intercalibration: individual absolute unit counts and mean values, coefficients of variation (CV% = standard deviation as percentage of the mean values) among professionals and among all participants. Taxa that were counted were laboratory strains of the following genera: *Selenastrum*, *Anabaena* and *Planktothrix*; Sele = *Selenastrum*, Anab1 = *Anabaena* cells, Anab2 = *Anabaena* trichomes, Plan1 = *Planktothrix* units of 25 µm, and Plan2 = *Planktothrix* trichomes.

| Counter no. | Sele | Anab1 | Anab2 | Plan1 | Plan2 |
|---|---|---|---|---|---|
| 1 | 64603027 | 42989047 | 8997708 | 12496816 | 5898497 |
| 2 | 70461235 | 37890346 | 7498090 | 9697529 | 5398625 |
| 3 | 70135779 | 35291008 | 5898497 | 8097937 | 5198675 |
| 4 | 80387645 | 35790881 | 6898242 | 12796740 | 5698548 |
| 5 | 65253939 | 47687850 | 7798013 | 10297376 | 3998981 |
| 6 | 55327529 | 54186195 | 9897478 | 16395823 | 5398625 |
| 7 | 69973051 | 32091824 | 4298905 | 11497071 | 5698548 |
| 8 | 64928483 | 44288716 | 9297631 | 12296867 | 6698293 |
| 9 | 79411277 | 35690907 | 7298141 | 13096663 | 5098701 |
| 10 | 65742123 | | | | |
| 11 | 73715796 | | | | |
| 12 | 72576700 | 35191034 | 5798523 | 17495543 | 5698548 |
| 13 | 72576700 | 29692435 | 5098701 | 9697529 | 4198930 |
| 14 | 80550373 | | | | |
| 15 | 70135779 | | | | |
| 16 | 63952114 | | | | |
| 17 | 74203980 | | | | |
| 18 | 77946725 | | | | |
| 19 | 69973051 | | | | |
| 20 | 77295813 | 45388436 | | 9597555 | |
| 21 | 64277571 | 39090041 | 6798268 | 8997708 | 3499109 |
| 22 | 63789386 | 29992359 | 4798777 | 8197911 | 4598828 |
| Professionals | | | | | |
| Mean | 69085444 | 40656308 | 7542523 | 11852536 | 5454166 |
| CV% | 10 | 18 | 23 | 20 | 13 |
| *N* | 11 | 9 | 9 | 9 | 9 |
| Non-professionals | | | | | |
| Mean | 71570745 | 35870861 | 5618835 | 10797249 | 4498854 |
| CV% | 8 | 18 | 16 | 35 | 20 |
| *N* | 11 | 5 | 4 | 5 | 4 |
| All | | | | | |
| Mean | 70328094 | 38947220 | 6952075 | 11475648 | 5160224 |
| CV% | 9 | 18 | 25 | 25 | 17 |
| *N* | 22 | 14 | 13 | 14 | 13 |

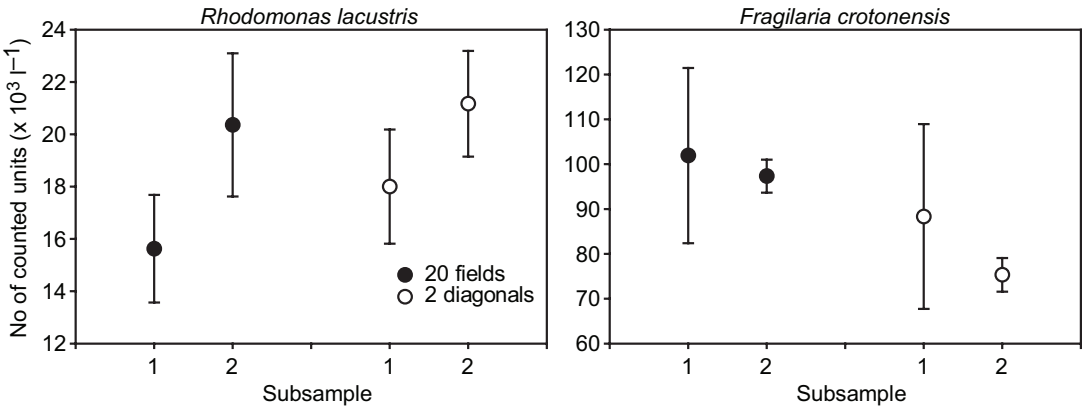


**Fig. 1**. The mean numbers (units $l^{-1}$ ± SD) of *Rhodomonas lacustris* and *Fragilaria crotonensis* in the two settled subsamples during the second intercalibrations representing repeatability of cell counts by Toini Tikkanen.

nals). For *Rhodomonas* cells the count from 20 fields gave a CV of 13% for both subsamples, and that from crossing diagonals was 12% for the first subsample and 10% for the second subsample. For *Fragilaria* cells corresponding CVs were 19% and 14% when enumerated from 20 fields and 23% and 5% when enumerated from crossing diagonals, respectively. The highest CV was probably due to uneven sedimentation in one of the three subsamples sedimented. The numbers of *Rhodomonas* ranged from 165 000 to 235 000 units $l^{-1}$ when counted from 20 fields and from 133 380 to 226 200 when counted from crossing diagonals. The corresponding values for *Fragilaria* cells were 690 000–1 100 000 and 808 080–1 993 400 units $l^{-1}$ (Fig. 1). This variability was thus considerably smaller than variability between different analysts counting subsamples of the same material. However, we also observed a statistically significant difference (Mann-Whitney *U*-test: $U = 3.5$, $P = 0.02$) between the two sedimented chambers in *Rhodomonas* counts. The CV was lower when more *Fragilaria* cells were counted from two diagonals, however, there were no significant differences (Mann-Whitney *U*-test) between the two methods.

## Discussion

During all three intercalibrations, thorough instructions were given for sample preparation, enumeration technique, and taxa to be counted. During the first intercalibration, participants were allowed to use their own modification of the Utermöhl method. To decrease the minimum variation, participants were given more detailed instructions, e.g. a minimum number of units to be counted during the second intercalibration. The maximum variation, however, increased within non-professional analysts but especially within professional analysts. When differences arising from subsampling and sedimentation were excluded in the third intercalibration, the variation within professional analysts decreased further but the variation also decreased within non-professional analysts. Despite this decrease, however, the inexperience of some participants caused unavoidable remaining variation in the results. Due to high overall variation within both professional and non-professional analysts, the differences between professional and non-professional analysts were not significant.

Most previous intercalibrations have harmonized marine phytoplankton analyses (e.g. Hobro and Willén 1977, Barinova *et al*. 1980, Hällfors and Niemi 1990, HELCOM 1997) and fewer intercalibrations have been concerned with freshwater phytoplankton (e.g. Rott 1981, Jensen and Søndergaard 1994, Veen *et al*. 2005). Our intercalibrations applied the Utermöhl technique, considered to be the most precise of existing methods and a common standard for phytoplankton analyses within EU (EN 15204 2006). The present study is limited in scope as compared with previous intercalibrations of freshwater phytoplankton (Rott 1981, Jensen and Sønder-

gaard 1994) deviating the following ways: First, entire samples from nature were not analysed; instead, only a few easily recognised species were considered. Second, calculations of the species biovolume were not performed; instead we enumerated only cell/colony numbers. Last, ultrasonically treated samples of colony forming cyanobacteria were not used, because ultrasonication complicates species identification, and because ultrasonication is not included in the new EU standard (EN 15204 2006). Despite these deviations our results are congruent with the earlier studies showing high variability (CVs) between laboratories and individual analysts, especially when natural samples are used (e.g. Hobro & Willén 1977, Barinova *et al*. 1980, Rott 1981, Niemi *et al*. 1985, Veen *et al*. 2005).

The exact quantitative composition of phytoplankton in a sample is difficult, if not impossible, to assess. Estimates of relative uncertainty therefore must be derived from phytoplankton counts by multiple persons. Quantitative uncertainty depends partly on the abundance of a specific taxon in a sample and partly on the amount of units counted (Lund 1958, Olrik *et al*. 1998). Because the volume of sedimented water is based on phytoplankton densities in water, changes in abundances affect the number of units enumerated (Eloranta 1978). This uncertainty of counts must be expressed for each taxon separately.

In our comparisons, counts of colonial species showed highest CVs between analysts. During the second intercalibration, the CV was high for *Desmodesmus* suggesting that some of the participants may have counted this bicellular species as single cells although instructions were given to count two-celled colonies. Moreover, high CV for *Radiocystis* suggests that the size of subcolonies was difficult to determine. Special attention was thus paid to the colony forming taxa that were counted, either as subcolonies estimated by the cell number (such as the cyanobacteria *Radiocystis*) or as single cells (the diatoms *Asterionella* and *Fragilaria*). Colonies consisting of several tens or hundreds of cells may give a distorted picture of the number of colonies if only the number of cells is enumerated. Therefore, a sufficient number of colonies (e.g. *Radiocystis geminata*), not cells, must thus be counted to reduce the CV. Some researchers have tried to solve the problem by counting ultrasonically treated samples, where the CV would have been much reduced (Jensen and Søndergaard 1994, Olrik *et al*. 1998). Even in this method, however, sufficient numbers of colonies may not be counted as the cells may originate from only a few large colonies, e.g. during HELCOM intercalibrations systematic errors arouse from low counts (HELCOM 1997). In the present study the extent of uncertainty (CV) decreased with increasing number of units counted. Our results confirm that the number of units counted has to be high enough to minimize the CV.

Even though special attention was paid to the less trained participants, our results reveal that the primary determinants of variation between analyses are the experience and carefulness of the participants as earlier noticed by Barinova *et al*. (1980) and Rott (1981). This variation, specifically, may originate from the failure to precisely follow instructions, individual modifications of the Utermöhl method, or differences in the optical quality of microscopes. Before phytoplankton results can be accepted as valid in an international context, analysts must thus undergo thorough training at a professional laboratory and intercalibrate their results with those of professional analysts.

Variability between natural phytoplankton samples can be high and further increase the variability between analysts (Hakala *et al*. 2002). In this study, the variability between natural subsamples counted by one analyst was as high as 20%, and even higher, up to 57%, between different analysts. This variability, thus, has to be considered when comparing data with the valid ecological class boundaries. The reliability and comparability of quantitative and qualitative phytoplankton data in assessment of the ecological classification of lakes and coastal waters depends as well on the quality of the work on phytoplankton and also very much on the person that works up the material. In future, this variability challenges the implementation of ecological classification of waters because data from human impacted waters analyzed by various laboratories is compared with reference data analyzed by environmental authorities.

The results of these intercalibration tests showed that it is necessary to elaborate a test

which excludes the variation between subsamples of natural phytoplankton to reliably compare true variability between analysts. Furthermore, a good knowledge of phytoplankton taxonomy is essential in order to correctly identify species for ecological classification. Therefore, in addition to the technical performance of phytoplankton enumeration, comparison tests for species identification are also needed.

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